# Inversa Dystrophic Epidermolysis Bullosa Is Caused by Missense Mutations at Specific Positions of the Collagenic Domain of Collagen Type VII

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## TO THE EDITOR

Inversa recessive dystrophic epidermolysis bullosa (RDEB-I) is a rare form of dystrophic epidermolysis bullosa (DEB) characterized by a peculiar course. Generalized blistering occurs in the neonatal period, which in early childhood heals with atrophic scars. The condition improves with age, subsiding to a predominance of flexural involvements in adults, mainly in inguinal, perineal, axillary, cervical, and submammary folds. The progressive amelioration of skin fragility is concomitant with the development of severe lesions of the oral, esophageal, anal, and genital mucosas (Fine *et al.*, 2008). As in the more recurrent forms of DEB, the bullae present a cleavage plane within the upper papillary dermis of the dermal–epidermal junction. RDEB-I has been associated with genetic mutations in the *COL7A1* gene, which codes for collagen type VII (Col7), the major component of the anchoring fibrils of the dermis (Christiano *et al.*, 1996). Patients with RDEB present mutations on both *COL7A1* alleles, and may thus either lack expression or express a mutated form of Col7, which, respectively, define the most severe Hallopeau–Siemens form or the milder clinical forms of the disease. On the other hand, patients with dominant DEB always express abnormal Col7 molecules, which harbor mutations that exert a dominant-negative effect on the protein. The fact that only seven patients with RDEB-I have been genotyped to date has hampered the establishment of a precise genotype–phenotype correlation. In this study, we report six new cases of RDEB-I and the identification of six genetic mutations in *COL7A1* that are, to our knowledge, previously unreported. These data, together with the information available in the literature, link RDEB-I to missense mutations at specific positions of the Col7 polypeptide, which allows for a hypothesis on their effect on the stability of the molecule, and provides hints on early diagnostic indications.

Supplementary Table S1 online summarizes the clinical features of the patients. Skin specimens of patients 1, 4, 5, and 6 were analyzed by immunohistochemistry using antibodies specific to the major components of the basement membrane of the dermal–epidermal junction (Charlesworth *et al.*, 2003). Epitope mapping detected a cleavage within the papillary dermis, which is the hallmark characteristic of DEB, and immunoreactive Col7 at the blister roof, with a reduced intensity of the signal in patients 4, 5, and 6. However, no correlation existed between the staining pattern and the clinical picture. All the other major antigens of the basement membrane zone were expressed at the blister roof. Direct DNA sequencing of genomic DNA identified nine genetic mutations in *COL7A1* gene, six of which, to our knowledge, are previously unreported (Figure 1). Patient 1 was homozygous for a missense mutation. Patients 2–6 were compound heterozygotes for a missense mutation and a premature termination codon inherited from heterozygous healthy carrier parents, which attests for the recessive inheritance of the condition, because the negative effect of the missense mutations is complemented by a wild-type *COL7A1* allele. In addition, the different premature termination codons found in compound heterozygosity clearly do not influence the clinical phenotype (Figure 1 and Supplementary Table S1 online).

The large constellation of genetic mutations identified in DEB has allowed for a relatively clear genotype–phenotype correlation between severe RDEB-HS and the homozygous or heterozygous combination of genetic mutations that hamper expression of the *COL7A1* alleles. However, a genotype–phenotype correlation still remains elusive for a number of DEB variants, including RDEB-I. Combining the results of the mutation analysis on the RDEB-I families identified so far (Figure 1a), the condition seems to be associated with missense mutations involving four arginine or four glycine, all falling within the carboxyl portion of the triple helix domain (THD) (Figure 1b). The THD domain is composed of repeating Gly–X–Y sequences that fold into a triple helix, which contains 19 imperfections/interruptions generating 20 distinct subdomains (COL1–COL20) that are highly conserved among species (Christiano *et al.*, 1994). The conservation of Gly residues in every third position of the amino-acid sequence is required for the tied packing of the triple helix in which glycines are placed in the center and the X and Y residues are exposed at the surface. Therefore, glycine substitutions highly destabilize the triple helix. In contrast to other collagens, the potential disruptive effect of glycine substitutions in Col7 is reduced by the presence of the short non-collagenous interruptions that are thought to provide conformational flexibility to the THD (Persikov *et al.*, 2004).

Identification of the novel mutation R2628W in our patient links RDEB-I with arginine substitutions involving

*Abbreviations: Col7, collagen type VII; DEB, dystrophic epidermolysis bullosa; RDEB-I, inversa recessive dystrophic epidermolysis bullosa*

**a**

| Case | Mutation | Exon | Nucleotide change | Consequence | Reference |
|---|---|---|---|---|---|
| 1 | G2775S<br>G2775S | 112 | 8323 G→A | Missense<br>Missense | This study |
| 2–3 | G1907D<br>R1340X | 68<br>34 | 5720–21 GA→AT<br>4018 C→T | Missense<br>PTC | This study |
| 4 | R2628W<br>Y444X | 106<br>10 | 7882 C→T<br>1332 C→A | Missense<br>PTC | This study |
| 5 | G2472D<br>1874del2 | 97<br>14 | 7415 G→A<br>1874del2 | Missense<br>PTC | This study |
| 6 | G2088R<br>R226X | 75<br>5 | 6262 G→A<br>676 C→T | Missense<br>PTC | This study |
| 7 | R2063G<br>R236X | 74<br>6 | 6187 C→G<br>706 C→T | Missense<br>PTC | Hovnanian *et al.* (1994) *Am J Hum Genet* 55: 289 |
| 8 | R2622W<br>2482delCT | 105<br>19 | 7864 C→T<br>2482delCT | Missense<br>PTC | Gardella *et al.* (2002) *J Invest Dermatol* 19: 1456 |
| 9 | G2775S<br>425 A/G | 112<br>3 | 8323 G→A<br>425 A→G | Missense<br>PTC | Zimmer *et al.* (2002) *Gastroenterology* 122: 220 |
| 10–11 | R2069C<br>425 A/G | 74<br>3 | 6205 C→T<br>425 A→G | Missense<br>PTC | Kahofer *et al.* (2003) *Pediatr Dermatol* 20: 243 |
| 12 | R2622W<br>R185X | 105<br>5 | 7864 C→T<br>553 C→T | Missense<br>PTC | Escámez *et al.* (2010) *Br J Dermatol*, in press |

**b**

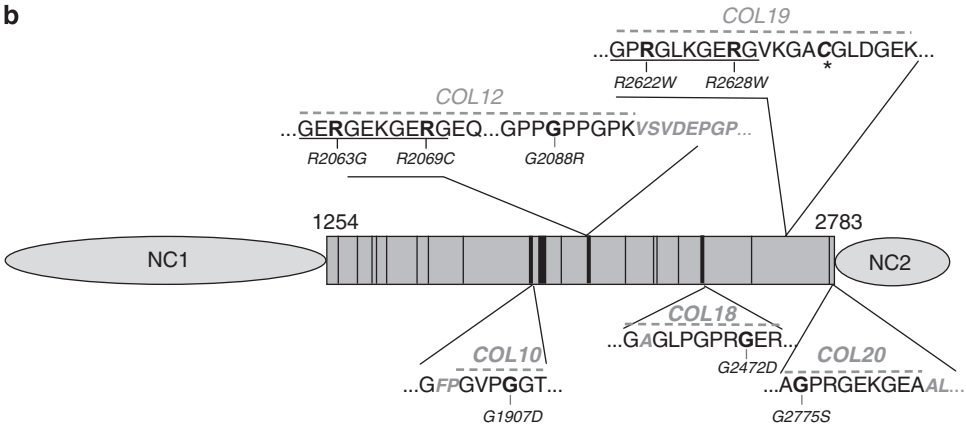


**Figure 1. Genetic mutations associated with inversa recessive dystrophic epidermolysis bullosa (RDEB-I).** (**a**) Genotype of the RDEB-I patients. (**b**) Representation of the collagen type VII (Col7) molecule, and localization of the genetic mutations associated with RDEB-I. Col7 is a homotrimeric adhesion molecule that harbors a 145-kDa central collagenous segment (THD) flanked by a large N-terminal (NC1) and a small C-terminal non-collagenous domain (NC2). Black bars indicate the 19 non-collagenic interruptions and imperfections of THD. The amino-acid sequence of the five collagenic domains (COL) affected by the arginine (bold R) or glycine substitutions (bold G) found in RDEB-I are reported. The non-collagenic interruptions are in small capitals in light gray italics, and the hydrophilic motifs $_{2061}$GE**R**GEKGE**R**$_{2069}$ and $_{2620}$GP**R**GLKGE**R**$_{2628}$ are underlined. Cysteine 2634, which mediates intramolecular interactions between Col7 monomers, is indicated (*).

two pairs of residues (R2063G/R2069C and R2622W/R2628W), each found at the Y position within the hydrophilic motifs $_{2061}$GE**R**GEKGE**R**$_{2069}$ and $_{2620}$GP**R**GLKGE**R**$_{2628}$, which are highly conserved among species (Figure 1 and Supplementary Figure S1). Specifically, residues R2622 and R2628 within subdomain COL19 juxtapose cysteine 2634, which is thought to mediate disulfide bonds with the non-collagenous domain (NC2) of another collagen VII molecule. The substitution of the large hydrophilic arginine residue by a large hydrophobic tryptophane is, therefore, likely to modify both the tertiary and quaternary structure of Col7 and the intermolecular interactions of Col7, with slightly weakened stability or folding of the molecule (Woodley *et al.*, 2008), which results in the very localized involvements observed in patient 4 (Supplementary Table S1 online). In comparison, residues R2063 and R2069 fall within subdomain COL12, which is considered crucial for the function of collagen VII (Christiano *et al.*, 1994). Substitution of a large arginine by either a small polar (glycine$_{2063}$) or a non-polar (cysteine$_{2069}$) residue, which preserves hydration of polypeptide $_{2061}$GE**R**GEKGE**R**$_{2069}$ by the surrounding aqueous environment, thus causes the peculiar inversa form of RDEB, whereas the disruptive substitution with a large hydrophobic

tryptophane results in severe RDEB-HS (Hovnanian *et al.*, 1994).

Interestingly, the four causative glycine substitutions associated with RDEB-I localize close to THD interruptions (Figure 1b). Mutations G1907D and G2088R disrupt the second and the second to the last Gly–X–Y repeats of subdomains COL10 and COL12, respectively, whereas mutations G2472D and G2775S disrupt the third and third to the last Gly–X–Y repeats of COL18 and COL20, respectively (Figure 1b). Besides mutation G1907D, no other glycine substitution has been reported in COL10, which adjoins the major 39-amino-acid non-collagenous hinge domain of the THD. Interestingly, the amino-acid sequence of COL10 is highly conserved among species, and RDEB dogs showing the hallmarks of RDEB-I harbor a genetic mutation (G1906A), which modifies the second Gly–X–Y repeat of COL10 (Baldeschi *et al.*, 2003). In addition, mutations G2472D and G2575S are the only glycine substitutions so far identified in COL18 and COL20, respectively. Mutation G2088R, which disrupts the penultimate Gly–X–Y of COL12, distinguishes itself from the other 33 missense mutations involving the 16 glycines in the middle of COL12 that generate DEB as either dominant (23 cases) or recessive (10 cases) inheritance (Dang and Murrell, 2008; Kern *et al.*, 2009). Recent studies showed that the effects of mutations in collagens are modulated by both the helix propensity of the surrounding amino-acid residues and the long-range molecular interactions of the triple helix (Xu *et al.*, 2008). The Col7 THD is organized into thermally stable and labile domains. Our observations suggest that the glycine substitutions localized at the edges of the collagenic THD subdomains induce a pathological phenotype characterized by a recessive inheritance and a thermosensitive pattern that is not observed in the case of Gly mutations that affect the central portions of the collagenic segments. Consistent with the typical aggravation of RDEB in several patients in summer, it has been recently shown that the glycine substitutions in Col7 may alter the thermal stability of the molecule (Fritsch *et al.*, 2009). In this respect, in RDEB-I, the predominant occurrence of cutaneous lesions in the mucosa and flexural area of the skin strengthens the hypothesis that in RDEB-I the localization of the amino-acid substitutions in specific THD domains correlates with the synthesis of a thermo-labile Col7 that is specifically less stable in the warmest areas on the patient's body. A localized distribution of the involved areas of the integument is observed in other skin conditions, including oculo-cutaneous albinism type 1, epidermolysis bullosa simplex, and the bathing suit ichthyosis, wherein it has been linked to temperature-sensitive amino-acid substitutions in the gene alleles associated with the condition (King *et al.*, 1991; Morley *et al.*, 1995; Oji *et al.*, 2006). Thus, the thermosensitive nature of the genetic mutations so far associated with RDEB-I could be directly confirmed by expressing mutant Col7 cDNAs in *COL7A1*-null keratinocytes following the experimental approaches recently reported by Fritsch *et al.* (2009).

In conclusion, the clinical identification of rare forms of inherited EB, including RDEB-I, in young patients is often difficult, and in several cases hampers prediction on the evolution of the condition. Our results suggest that in DEB patients having amino-acid substitutions affecting either the arginine residues within the hydrophilic highly conserved motifs of the Col7 THD domain or the glycines at the borders of the collagenic subdomains, the diagnosis of RDEB-I should be considered, and, in turn, a simple advice to alleviate the associated discomforts in patients would be to keep the temperature of the involved integument as low as possible, and consume cold food and beverages.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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# Changes in the Ceramide Profile of Atopic Dermatitis Patients

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## TO THE EDITOR

We wish to report the characteristic differences that have been identified between the ceramide (CER) profiles of atopic dermatitis (AD) patients and those of healthy individuals.

AD is characterized by impaired stratum corneum (SC) functions, which can be indicated by either an increase in transepidermal water loss (TEWL) or a decrease in water-holding function (capacitance). On the other hand, a close relationship between the impaired SC functions and CERs has been reported (Elias, 1983; Melnik *et al.*, 1988; Yamamoto *et al.*, 1991; Motta *et al.*, 1993, Paige *et al.*, 1994; Di Nardo *et al.*, 1998; Matsumoto *et al.*, 1999; Macheleidt *et al.*, 2002; Cho *et al.*, 2004). More recent research has attempted—but failed—to uncover the diagnostic potential of CER profiling for skin pathologies, including AD (Farwanah *et al.*, 2005).

Human SC CERs can be divided into 11 groups according to their fatty acid and sphingoid structures (Masukawa *et al.*, 2008), as shown in Figure 1a: CER[NDS] contains non-OH fatty acids [N] and dihydrosphingosines [DS]; CER[NS] contains [N] and sphingosines [S]; CER[NH] contains [N] and 6-hydroxy sphingosines [H]; CER[NP] contains [N] and phytosphingosines [P]; CER[ADS] contains α-OH fatty acids [A] and [DS]; CER[AS] contains [A] and [S]; CER[AH] contains [A] and [H]; CER[AP] contains [A] and [P]; CER[EOS] contains ester-linked fatty acids, and ω-OH fatty acids [EO] and [S]; CER[EOH] contains [EO] and [H]; and CER[EOP] contains [EO] and [P]. These classes have been further subdivided into species by chain length. To date, we have identified 350 species in human SC, using normal-phase liquid chromatography coupled with electrospray ionization–mass spectrometry (Masukawa *et al.*, 2008, 2009; it is worth noting that the former was used to separate CERs into classes based on the polarities). Using the same procedure, we obtained an exhaustive CER profile of the skin of AD patients and healthy individuals to find a characteristic difference between their profiles.

The study was conducted according to the Declaration of Helsinki Principles. The protocol was approved by the ethics committees of the Kao Corporation and Dokkyo Medical University. Informed consent to participate in this study was obtained from the patients. Eight subjects with mild levels of AD (four men and four women; age range, 16–37; mean, 28) and seven healthy individuals with no history of skin disorder (four men and three women; age range, 25–37; mean, 31) were enrolled in this study. First, we evaluated TEWL and capacitance, respectively, using a Tewameter TM 210 and a Corneometer CM 825 (Courage + Khazaka Electronic GmbH, Cologne, Germany) in the affected and unaffected sites on the forearm skin of AD patients and on the forearm skin of healthy individuals. After confirming that TEWL and capacitance were significantly higher and lower, respectively, in the affected sites of the AD patients as compared with those of the unaffected sites of the AD patients and healthy individuals (Figure 1b and c), we sampled SC specimens from these skin sites by performing tape stripping 10 times. CERs were in turn extracted from these specimens and analyzed with an Agilent 1100 Series LC/MSD single-quadrupole system equipped with an electrospray ionization source, ChemStation software, a 1,100-well-plate autosampler (Agilent Technologies, Palo Alto, CA), and an Inertsil SIL 100A-3, 1.5 mm i.d. × 150 mm column (GL Science, Tokyo, Japan). These procedures are detailed in our previous reports (Masukawa *et al.*, 2008, 2009).

The levels of total CER as well as those of CER[NH], CER[NP], CER[EOS], CER[EOH], and CER[EOP] classes were found to be significantly lower in the affected sites of AD patients as compared with the normal skin sites of healthy individuals (Figure 1d and e). These findings are consistent with previous findings (Imokawa *et al.*, 1991; Yamamoto *et al.*, 1991; Di Nardo *et al.*, 1998; Bleck *et al.*, 1999; Macheleidt *et al.*, 2002). In addition, this study revealed that the expression levels of CER[AS] were significantly higher in the affected sites of AD patients as compared with the normal sites of healthy individuals.

*Abbreviations: AD, atopic dermatitis; CER, ceramide; SC, stratum corneum; TEWL, transepidermal water loss*